Riboflavin-Catalyzed Dehydrogenation of Dihydrophthalates in the Dark*

Gerald D. Weatherby† and Daniel O. Carr

ABSTRACT: Riboflavin catalyzes the dehydrogenation of the esters of 1,2-dihydrophthalic acid at pH >9 in the dark. The product of the dehydrogenation of methyl hydrogen *cis*-1,2-dihydrophthalate has been shown to be methyl hydrogen phthalate. The reaction is due to a bimolecular collision of riboflavin with the anionic forms of the esters. Although complexes are formed between the reductants and the riboflavin, as adjudged by fluorescence quenching, these complexes apparently are not involved in the reaction nor do they inhibit the reaction. The effect of pH and ionic strength were in keeping with the polar anionic forms being the reactive species.

It should be emphasized that the anionic form of riboflavin is the predominant form in the pH range studied. Consequently, the ionization of riboflavin does not affect its ability to accept electrons. The enthalpy of activation for the dehydrogenation of the *cis*-half-ester was found to be +6.64 kcal/mole and the entropy was −54 cal/deg per mole.

Flavoenzymes catalyze redox reactions involving an alkene linkage, α,β to a carbonyl group. As pointed out in the previous paper (Weatherby and Carr, 1970), our studies have been directed toward developing a model system that might yield some insight into the mechanism of such reactions. It was reported that riboflavin can be used to bring about a photocatalyzed oxidative decarboxylation. Because of problems associated with photoreactions and because of the difficulty in making direct correlations to physiological systems, other conditions were used to produce a flavin-catalyzed dehydrogenation in the dark.

Only a few other nonphotocatalyzed, nonenzymic flavin-catalyzed dehydrogenations have been reported. These have involved NADH or its analogs (Singer and Kearney, 1950; Suelter and Metzler, 1960; Fox and Tollin, 1966) or dihydrolipoic acid (Gascoigne and Radda, 1967). The dehydrogenations reported here have a direct relationship not only to fatty acyl-CoA dehydrogenases and succinic acid dehydrogenase, but also to the dehydrogenases that oxidize dihydro- and hexahydrobenzenoid compounds (Gibson *et al.*, 1968; Young *et al.*, 1969; Babior and Bloch, 1966).

Experimental Section

Materials. This *cis*- and *trans*-1,2-dihydrophthalic acids were prepared as described by Baeyer (1892), and the esters were prepared as described previously (Weatherby and Carr, 1970). All other compounds were commercial products and were used as supplied.

Methods. The reactions were conducted in Thunberg tubes with 1-cm spectrophotometer cells attached to the bottom. The flavin (3×10^{-5} M) and the buffer (0.5 ionic strength) were placed in the tube, and the reductant (methyl hydrogen *cis*-1,2-dihydrophthalate, dimethyl *trans*-1,2-dihydrophthalate, or dimethyl *cis*-1,2-dihydrophthalate) was placed in the side arm. The Thunberg tubes were evacuated for 2 min to remove oxygen from the system and were placed in a water bath to equilibrate for 10 min. The reaction was started by tipping in the contents of the side arm, and the course of the reaction was followed by observing the decrease in the flavin absorbance at 445 nm. The reductants were always used in at least tenfold excess of the flavin so that pseudo-first-order analyses could be applied to the data. The effects of pH, ionic strength, heat, and concentration of the reductant on the reaction were studied by this procedure.

The product of the riboflavin oxidation of methyl hydrogen *cis*-1,2-dihydrophthalate in the dark was isolated by the following scheme. A 10-ml reaction solution which was 10^{-5} M in riboflavin, 10^{-3} M in dihydrophthalate, and 0.1 M in phosphate buffer at pH 11.0 was allowed to stand in the dark at 30°. After 15 min, the pH of the solution was adjusted to pH 1.0 by using 2 M hydrochloric acid. The acidified solution was extracted three times with 3 ml of ether. The ether extracts were combined and evaporated to about 0.2 ml and spotted on silica gel thin-layer chromatography plates. The solvent composition for the thin-layer chromatography was 80 ml of chloroform, 20 ml of cyclohexane, and 10 ml of glacial acetic acid (Stahl, 1965).

Interaction of the flavin with methyl hydrogen *cis*-1,2-dihydrophthalate was determined at pH 9 on the reaction from the amount of quenching of the flavin fluorescence. Solutions (1.70 ml) which contained 0.20 ml of 1.5×10^{-4} M riboflavin and varied amounts of neutralized dihydrophthalate and water were prepared in 1-cm fluorescence cells in the dark. The cells were placed in a spectrofluorometer and 0.30 ml of the buffer (pH 9) was added immediately by using an adder mixer. This method was necessary to avoid the effect of isomerization of the esters. Dissociation constants were calculated from the amount of quenching.

Results

Reduction of the Flavins. Dimethyl *cis*-1,2-dihydrophthalate, dimethyl *trans*-1,2-dihydrophthalate, and methyl hydrogen

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† Predoctoral fellow of the United States Public Health Service.

cis-1,2-dihydrophthalate reduce riboflavin or 3-methyllumiflavin in the dark under anaerobic conditions at pH 11.0. The reduction of the flavin was reversible since admission of air into the Thunberg tube resulted in quantitative return of the flavin absorbance at 445 nm. Control experiments which lacked only the esters of the dihydro acids gave no reduction of the flavin absorbance at 445 nm.

Isolation of the Products. The isolation of the product of the oxidation of the *cis*-half-ester was conducted as described in the Experimental Section. The chromatographic R_F values of the components of the reaction mixture indicated that a dehydrogenation had occurred. Only two migrating components could be found on the thin-layer plates. These components had R_F values which corresponded to the *cis*-half-ester and methyl hydrogen phthalate (Weatherby and Carr, 1970). A component was present in the reaction mixture which did not migrate in the solvent system which was used, but remained at the origin. This material is felt to be the by-product of a base-catalyzed side reaction of the *cis*-half-ester. Significant changes are observed in the spectra of the *cis*-half-ester when it is allowed to stand in solution above pH 9.0. This side reaction is slow compared with the reduction of the flavin and does not result in the formation of a benzenoid system as demonstrated by the low absorbance in the 220–230-nm region. The product of the flavin oxidation of the *cis*-half-ester is therefore methyl hydrogen phthalate.

Elution of the thin-layer plate in the region which corresponded to methyl hydrogen phthalate, resulted in a solution whose spectrum was identical with that of authentic methyl hydrogen phthalate.

The products of the flavin oxidations of the dimethyl esters of *cis*- and *trans*-1,2-dihydrophthalate have not been isolated. However, considering their similarity to the *cis*-half-ester, it is reasonable to predict that the product would be dimethyl phthalate in both cases.

Due to the base-catalyzed side reactions which occur with the dihydrophthalate esters, it was not possible to determine the stoichiometry for these oxidations based upon spectral analyses, as was done previously for the photocatalyzed reactions (Weatherby and Carr, 1970).

Order of the Reactions. Using the procedure described in the Experimental Section, the change in the flavin absorbance at 445 nm was followed as a function of time. When the log of the fraction of flavin remaining was plotted *vs.* time, a straight line was obtained. This indicated that the reaction was first order with respect to the flavin concentration. The order of the reaction with respect to the *cis*-half-ester was determined by measuring the pseudo-first-order constants for flavin reduction at several *cis*-half-ester concentrations. When a plot of log k_1' *vs.* $-\log$ [dihydrophthalate] was made, a straight line with a slope of -1.0 was found indicating that the reaction is first order with respect to the *cis*-half-ester. These results indicate that this reaction is bimolecular.

The data which were used to determine the order of the reaction with respect to the *cis*-half-ester concentration were replotted in a double-reciprocal form. A plot of $1/k_1'$ *vs.* 1/(*cis*-half-ester concentration) yielded a straight line which passed through the origin. This demonstrates that no reactive complex is formed between the flavin and the *cis*-half-ester, and the reaction follows collision kinetics which is in contrast to that found for the photocatalyzed reactions involving the same compounds (Weatherby and Carr, 1970).

Complex Formation. Even though the oxidation of the *cis*-half-ester by riboflavin in the dark follows collision kinetics, a fluorescence quenching complex is formed between these two reactants. The dissociation constant for this fluorescence quenching complex was 5.6×10^{-2} M.

No inhibition of the rate of oxidation of the *cis*-half-ester in the dark was observed at concentrations of the *cis*-half-ester where quenching of the riboflavin fluorescence occurred. This result would be expected since the reaction is not photocatalyzed.

Effect of Ionic Strength. The riboflavin oxidation of the *cis*-half-ester was markedly affected by the ionic strength of the reaction solution. Figure 1 shows that as the ionic strength was increased from 0.03 to 0.30, there was a fourfold increase in the second-order rate constant for the reaction. Above 0.30 ionic strength, there was essentially no effect. These results are compatible with a reaction mechanism which involves polar intermediates which are subject to stabilization by interaction with a polar reaction medium.

Effect of Temperature. The riboflavin oxidation of the *cis*-half-ester at pH 9.0 in the dark was conducted at various temperatures. The second-order rate constants which were obtained from these experiments were converted to pseudo-first-order constants by multiplying each second-order constant by a riboflavin concentration of 2×10^{-5} M. This riboflavin concentration is approximately that used experimentally, and was chosen for the convenience of calculation. Analyses of the data using the Eyring equation (Wynne-Jones and Eyring, 1935) revealed a $\Delta H^{\ddagger}$ of $+6.64$ kcal/mole and a $\Delta S^{\ddagger}$ of -54 cal/deg per mole.

Effect of pH. The effect of pH on the riboflavin oxidation of methyl hydrogen *cis*-1,2-dihydrophthalate is shown in Figure 2. These results indicate that the reaction is greatly enhanced as the pH is increased above pH 9.0. The hydrogen which is attached to the carbon which is α to the ester functional group would have a pK_a in the pH range where the marked effect on the reaction rate is observed. If the monoionized reductant is the reactive species, then a plot of $[H^+]$ *vs.* k_2 should yield a straight line with an intercept equal to the dissociation constant. Such a plot was linear and revealed a pK_a of 11.0.

Since no inhibition was noted, the ionization of the 3 position of riboflavin ($pK_a = 10$) apparently does not influence the reaction. This was confirmed by repeating the determination of the pK_a for the rate-determining ionization using 3-methyllumiflavin as the oxidizing agent. The data again indicated that single ionization with a pK_a of 11.0 was involved in the reaction.

Also, maximal second-order rate constants could be calculated based upon the total ionization of the reductant. These values were 2.1×10^4 M^{-1} min^{-1} and 3.6×10^4 M^{-1} min^{-1} for riboflavin and 3-methyllumiflavin, respectively. The close agreement between the pK_a and k_2 (max) values shows that no great difference exists between riboflavin and 3-methyllumiflavin as oxidizing agents in this system.

When a similar analysis was conducted on the pH effect of the riboflavin oxidation of the dimethyl *trans*-1,2-dihydrophthalate in the dark, it was found that the ionization of two hydrogens with pK_a's of 11.2 were involved in the reaction. This suggests that the diionized form of the diester is the reactive species in the oxidation. Studies on these oxidations at pH >11.0 have not been possible due to the rapidity of the

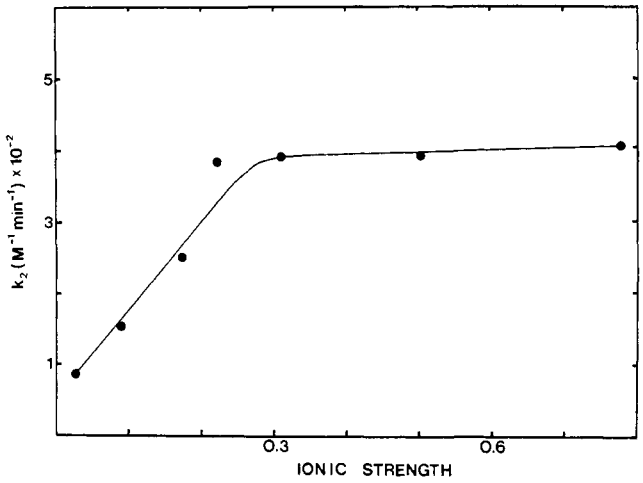

FIGURE 1: Effect of ionic strength on the flavin oxidation of methyl hydrogen-*cis*-1,2-dihydrophthalate in the dark. Measurements were conducted at pH 9.0 in carbonate buffer.

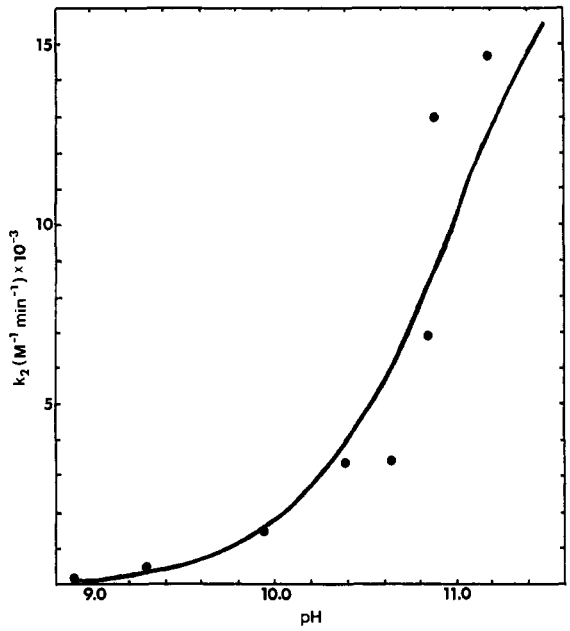

FIGURE 2: pH dependency of the riboflavin oxidation of methyl hydrogen *cis*-1,2-dihydrophthalate in the dark. The curve has been drawn for a titration curve with a pK_a of 11.0.

reaction and due to the competing base-catalyzed side reactions. The pH profile for the dimethyl *cis*-1,2-dihydrophthalate has not been determined because the base-catalyzed side reaction proceeded much faster than the reduction of the flavin, even at pH 10.

It was possible to obtain a second-order rate constant at pH 9.3 for oxidation of dimethyl *cis*-1,2-dihydrophthalate, and this value was considered reliable. At this low pH, the base-catalyzed side reaction was slow compared with the riboflavin reduction. Table I presents a comparison between the second-order rate constants at pH 9.3 for the riboflavin oxidation of the three esters studied in these dark reactions. The order of reactivity for these esters is: *cis*-diester $\gg$ *cis*-half-ester > *trans*-diester.

Discussion

The results of this study clearly establish that the flavin oxidation of methyl hydrogen *cis*-1,2-dihydrophthalate yields methyl hydrogen phthalate. The chromatographic R_F value of the product, and the spectrum of the product after isolation by thin-layer chromatography provide conclusive evidence for product identification. The products of the flavin oxidations of dimethyl *cis*-1,2-dihydrophthalate and dimethyl *trans*-1,2-dihydrophthalate have not been isolated.

The oxidation of the *cis*-half-ester has been found to be a bimolecular reaction which follows collision kinetics. The thermodynamic data support this conclusion since the positive enthalpy of activation indicates that an increased temperature would increase the rate of the reaction by increasing the number of collisions which would have sufficient energy to result in product formation. This is in contrast to the photocatalyzed reactions (Weatherby and Carr, 1970) where a reactive complex was formed prerequisite to the redox reaction.

The effect of pH and ionic strength upon the oxidations of the 1,2-dihydrophthalate esters in the dark suggests that the reactions proceed *via* polar intermediates. The ionization of a single hydrogen with a pK_a of 11.0 was found to be involved in the flavin oxidation of the *cis*-half-ester, and the ionization of two hydrogens with pK_a's of 11.2 was found to be necessary for the oxidation of the *trans*-diester. This suggested that the monoionized *cis*-half-ester and the diionized diester were the reactive species in the redox reaction. Due to the rapid base-catalyzed side reaction, it was not possible to determine the degree of ionization for the reactive species of the *cis*-diester. However, the rates of the riboflavin oxidations of the three 1,2-dihydrophthalate esters at pH 9.3 were compared. The order of reactivity was found to be: *cis*-diester $\gg$ *cis*-half-ester > *trans*-diester. These results were felt to reflect three phenomena: (a) The *cis* esters were better reductants because one side of the cyclohexadiene ring was free of bulky substituents, and this favored a necessary interaction between the essentially planar cyclohexadiene and flavin. The *trans*-diester would have steric hindrance on both sides of the cyclohexadiene ring. (b) The *cis*-diester is by far the best reductant because of the increased acidity of the second α hydrogen. (c) The rate of the *cis*-half-ester oxidation is slower because the carboxyl anion is repelled somewhat by the flavin anion.

The finding that there is no great difference between riboflavin and 3-methyllumiflavin in the pH range studied is somewhat surprising since one could expect that the full

TABLE 1: Second-Order Rate Constants for the Riboflavin Oxidations of the Methyl Esters of the 1,2-Dihydrophthalates.

Reductant	k_2 at pH 9.3 (M^{-1} min^{-1})
Dimethyl *cis*-1,2-dihydrophthalate	9.31×10^3
Methyl hydrogen *cis*-1,2-dihydrophthalate	4.20×10^2
Dimethyl *trans*-1,2-dihydrophthalate	1.63×10^2

negative charge on the ionized riboflavin might decrease its tendency to accept electrons in a redox reaction. The fact that no major difference is observed suggests that the negative charge on the riboflavin is isolated on the 3 position and does not greatly influence the electron-accepting centers of the flavin molecule. This suggestion is supported by the observation that ionization of the 3 position of riboflavin has little effect on the ultraviolet spectrum of the riboflavin (Ehrenberg and Hemmerich, 1968).

It is tempting to attribute the dehydrogenation of the *cis*-half-ester to a hydride-transfer mechanism. The ionization of the hydrogen which is α to the ester function would generate a carbanion which might be expected to displace a hydride ion to the flavin. However, in the *cis*-half-ester the carbanion and hydrogen to be displaced as a hydride ion are *cis* to each other. It is reasonable to predict that such a base-catalyzed hydride elimination would proceed by a mechanism comparable with base-catalyzed dehydrohalogenations. It has been shown that base-catalyzed dehydrohalogenations proceed by a *trans* elimination, and if the halogen is *cis* to the hydrogen to be eliminated, the reaction proceeds poorly (Fieser and Fieser, 1961). Therefore, it is not likely that the dehydrogenation proceeds by simple hydride displacement.

A more reasonable mechanism would be for the collision of the ester carbanion with the flavin to result in the transfer of the electrons of the carbanion to the flavin. The incipient carbonium ion which would be formed would be neutralized by the ionization of the hydrogen which is α to the free carboxyl group. In effect, this would be a concerted *cis* elimination. This mechanism is supported by the fact that the *cis*-diester is the most reactive isomer which was studied. The increased acidity of the second α hydrogen of the *cis*-diester, when compared with the acidity of the hydrogen which is α to the free carboxyl group of the *cis*-half-ester, would explain this greater reactivity. With both *cis*-esters, the hydrogens involved in the dehydrogenation are on the side of the cyclohexadiene ring which is free from steric hindrance due to the ester functional groups. This allows a close interaction between the two essentially planar ring systems. The actual electron transfer mechanism which has been described could also apply to the *trans*-diester. A slower rate was observed for this isomer due to the steric hindrance of the ester functions. Both sides of the cyclohexadiene ring would have bulky ester functions which could decrease the ability of this ester to achieve the necessary ring–ring interaction which would result in electron transfer.

This mechanism is actually based on the ability of the carbanion to donate its electrons to the flavin. The transition state could therefore be expected to resemble an alkylated flavohydroquinone. Heterolytic cleavage of the carbanion–flavin interaction in the reaction would result in the transfer of electrons from the reductant to the flavin. There is evidence available that anions do interact with flavins to give the appearance of reduction of the flavins. Swoboda and Massey (1966) found that glucose oxidase from *Aspergillus niger* interacted with bisulfite to give a spectrum which closely resembled the reduced enzyme. Additionally, high concentrations of bisulfite (0.2–1.4 M) resulted in the bleaching of the 445 nm absorbance of FMN. More recent studies (Müller and Massey, 1969) have shown this effect is due to adduct formation. It has been found in this laboratory that 1 M KCN interacts with 3-methyllumiflavin at pH 11.0 to yield a spectrum very similar to that of reduced flavin. It is felt that the evidence strongly supports the interaction of the carbanion of the esters as the mechanism for the transfer of electrons to the flavin in this system.

The base-catalyzed reaction of the proposed alkylated flavohydroquinone which was formed in the irreversible photoreduction of flavin by the *cis*- and *trans*-diester (Weatherby and Carr, 1970) suggests a different mechanism for the dark reactions. Once a true covalent bond has been formed to produce the alkylated flavohydroquinone, only the *trans* compound can undergo the base-catalyzed displacement reaction. This suggests that the displacement reaction proceeds by a *trans* elimination. Since the *cis*- esters are the most reactive in the dark reaction, it is suggested that the transition state which may resemble an alkylated flavohydroquinone does not result in the formation of an actual alkylated intermediate with a covalent carbon–flavin bond.

The biological implication of the mechanism suggested for these dark reactions is obvious. Up until now, the only single product carbon–carbon bond oxidations have been photocatalyzed reactions (Carr, 1961; Weatherby and Carr, 1970). With the present oxidations occurring in the dark, more direct analogies can be drawn to enzymic reactions. The presence of the guanidino group of arginine and ϵ-amino group of lysine in proteins provide suitably basic groups to ionize hydrogens with pK_a's around 11.0. It is suggested that flavoenzymes such as the acyl-CoA dehydrogenases or succinic acid dehydrogenase may have mechanisms which involve the ionization of a hydrogen on the substrate to generate a carbanion, with the redox reaction resulting from an interaction of the carbanion with the flavin prosthetic group.

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